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Cytoplasmic Localization Is Important for Transcription Factor Nuclear Factor-κB Activation by Hepatitis C Virus Core Protein through Its Amino Terminal Region

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We previously reported that hepatitis C virus core protein (core) activates the transcription factor nuclear factor-κB (NF-κB) when expressed transiently. In the present study, we investigated the relationship between the NF-κB activation capacity and subcellular localization of the core. By changing the subcellular localization of the C-terminally truncated core from the nucleus to the cytoplasm, NF-κB was activated. In addition, NF-κB activity was augmented by forcing the mutated core to move to the endoplasmic reticulum. It was also suggested that the region from aa 21 to 80 of the core is involved in the activation of NF-κB. © 2001 Academic Press

Key Words: hepatitis C virus; NF-κB; endoplasmic reticulum; subcellular localization.

INTRODUCTION

Hepatitis C virus (HCV), which has been identified as the major causative agent of non-A, non-B hepatitis (Choo *et al.,* 1989; Kuo *et al.,* 1989), is classified as a member of the *Flaviviridae* family (Choo *et al.,* 1991; Kato *et al.,* 1990; Takamizawa *et al.,* 1991). HCV has a positive-strand RNA genome consisting of an open reading frame (ORF) composed of over 9000 nucleotides. This RNA genome encodes a large precursor polyprotein, which is cleaved by the host and viral proteases to generate at least 10 functional viral proteins: core, envelope (E)-1, E2, p7, nonstructural protein (NS)-2, NS3, NS4A, NS4B, NS5A, and NS5B (Grakoui *et al.,* 1993; Hijikata *et al.,* 1991, 1993; Lin *et al.,* 1994; Mizushima *et al.,* 1994b). Persistent infection of HCV has been considered closely related to chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Alter *et al.,* 1989; Choo *et al.,* 1989; Kuo *et al.,* 1989; Liang *et al.,* 1993; Tanaka *et al.,* 1991). Although the pathogenesis of these HCV-related diseases is still unknown, studies have suggested a contribution by HCV proteins to the development of these diseases.

Recently, it was reported that the core protein, which is considered a viral structural protein processed from the N-terminal region of the HCV precursor polyprotein (Grakoui *et al.,* 1993; Hijikata *et al.,* 1991), has the potential to transform rat embryo fibroblasts, Rat-1 cells, NIH3T3 cells, and BALB/3T3 cells, although the coproduction of oncogenic Ras protein was required for the transformation in some cases (Chang *et al.,* 1998; Ray *et al.,* 1996a; Tsuchihara *et al.,* 1999). It was also reported that transgenic mice expressing the core developed hepatocellular carcinoma after the age of 16 months (Moriya *et al.,* 1998). These observations prompted the idea that the core has oncogenic potential and is closely related to hepatocarcinogenesis, when this viral protein is constitutively expressed in liver cells. In general, however, cells which are infected with a virus and producing viral proteins are believed to be attacked by the host immune system. Recent studies have suggested that apoptosis induced by cytotoxic T lymphocytes plays an important role in the clearance of infected cells (Moretta, 1997; O'Brien, 1998). As chronic infection is a characteristic feature of HCV, the possibility exists that HCV-infected cells become resistant to apoptosis induced by the host immune system. Several studies on the relationship between apoptosis and HCV proteins have been reported (Fujita *et al.,* 1996; Marusawa *et al.,* 1999; Ray *et al.,* 1996b, 1998; Ruggieri *et al.,* 1997; Zhu *et al.,* 1998). Recently, we found that cultured cells transiently expressing the entire ORF of the HCV genome became resistant to Fas- and tumor necrosis factor α-mediated apoptosis and that the core among HCV proteins was responsible for this effect (Marusawa *et al.,* 1999). We further suggested that the anti-apoptotic effect of the core was related to the activation of the transcription factor nuclear factor κB (NF-κB) by this viral protein in some cell lines.

We and others reported that the core showed potential to activate NF-κB in HepG2, MCF-7, Jurkat, and HeLa

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cells transiently producing this protein (Kato *et al.,* 2000; Marusawa *et al.,* 1999; Tai *et al.,* 2000). On the other hand, it was also reported that NF-κB activity in the cell lines which were established as permanent transfectants of the core was variable (Shrivastava *et al.,* 1998; You *et al.,* 1999; Zhu *et al.,* 1998). Although the effect of long-term production of the core on NF-κB activity remains to be clarified, the transiently produced core protein is likely to have the potential to activate NF-κB. NF-κB is known to be involved in a variety of physiological events, for example, cell proliferation, differentiation, survival, and inflammatory response (Gerondakis *et al.,* 1999; May and Ghosh, 1998). Therefore, activation of NF-κB by the core seems to contribute to the pathogenesis of HCV.

Previously, we have reported that the core with a truncated C-terminal region lost its ability to activate NF-κB (Marusawa *et al.,* 1999). We also found that the subcellular localization of this truncated core was mainly confined to the nucleus, while the wild-type core which was primarily translated as a polypeptide of 191 amino acids was located in the cytoplasmic perinuclear region (Marusawa *et al.,* 1999). However, it was unclear whether the NF-κB activation potential was lost through alteration of the subcellular localization or deletion of the active site. As it was reported that a certain molecule of the wild-type core is located in the nucleus (Yasui *et al.,* 1998), it has been unknown whether the core activates NF-κB in the perinuclear region of the cells. Therefore, in this study, we tried to analyze the molecular mechanism of NF-κB activation by the HCV core. Here, we report a correlation between the subcellular localization and NF-κB activation capacity of the core and the region of the core essential for the activity.

RESULTS

Cytoplasmic localization of the core is important for its NF-κB activation capacity

Our previous results indicated that the core activates transcription factor NF-κB when this protein is transiently expressed in several human cell lines, including HepG2 and MCF-7 cells (Marusawa *et al.,* 1999). To examine whether NF-κB is also activated in murine-derived cells, the expression plasmids, pCMV-Core, pCMV-ΔCore173, pCMV-ΔCore151, and pCMV-ΔCore100, encoding 1–191 aa (C191), 1–173 aa (C173), 1–151 aa (C151), and 1–100 aa (C100) of the core, respectively, were transfected into NIH3T3 cells. Subsequently, NF-κB activity in the transfected cells was evaluated by dual-luciferase reporter assay. As shown in Fig. 2A, we found a seven- to eight-fold augmentation of relative luciferase activity in the cells producing either C191 and C173, compared to that in the cells transfected with an empty vector, pKS+/CMV, as a negative control (Fig. 1A, lanes 1–3). The observed activation of reporter gene expression was likely to be NF-κB-specific because the transcription from the reporter plasmid whose promoter contained the mutated element of the NF-κB binding sequence was not affected by any core products (data not shown). In contrast, the relative luciferase activity of the cells transfected with pCMV-ΔCore151 and pCMV-ΔCore100, which encoded the core with larger deletions in the C-terminal region, was not augmented (Fig. 1A, lanes 1, 4, and 5). As the production levels of these core derivatives were confirmed to be almost equal by immunoblotting analysis (Fig. 1B, lanes 2–4), it was likely that the C-terminal region of the core is required for NF-κB activation. These results were consistent with those of our previous experiments using HepG2 and MCF-7 cells (Marusawa *et al.,* 1999). This suggested that the core activates NF-κB in cells irrespective of the mammalian species. Then, to see whether the C-terminal region of the core itself has the NF-κB activation capacity, we examined whether the transfection of pCMV-ΔCore(101–191) for expression of the C-terminal part of the core caused NF-κB activation in NIH3T3 cells. As shown in Fig. 1A, we did not observe any enhancement of the relative luciferase activity in the cells transiently producing this protein (Fig. 1A, lane 6). This indicated that only the C-terminal region of the core is incapable of activating NF-κB.

Next, we analyzed the subcellular localization of each C-terminally deleted core protein by indirect immunofluorescence microscopy. As shown in Fig. 1C, when C191 was produced, this protein was found to be located around the perinuclear region in the cytoplasm (Fig. 1Ca). And C173 was detected in both the nucleus and the cytoplasm (Fig. 1Cc). However, the subcellular localization of C151 and C100 was quite different from that of C191 and C173. These deletion mutant core proteins were mainly detected in the nucleus (Figs. 1Ce and 1Cg). The results were similar to those of our previous study and others in which experiments were carried out using human cell lines (Barba *et al.,* 1997; Marusawa *et al.,* 1999). Given the above results, it is highly likely that cytoplasmic localization of the core is important for activation of NF-κB. Therefore, we examined whether NF-κB activity is enhanced when the subcellular localization of C151, which did not activate NF-κB, is changed from the nucleus to the cytoplasm by addition of several modifications to this protein as follows: (1) fusion with a peptide containing the nuclear export signal (NES), (2) fusion with an uncleavable peptide with a signal sequence at the C-terminal end, (3) introduction of a point mutation into the nuclear localization signal (NLS) of the core.

For the construction of the NES fusion protein, we used the NES of the human T-cell leukemia virus type-I (HTLV-1) Rex protein and fused it at the C-terminus of C151 (Bogerd *et al.,* 1995). Since NES is known to be involved in active transport from the nucleus to the cytoplasm (Turpin *et al.,* 1999; Wen *et al.,* 1995), it was

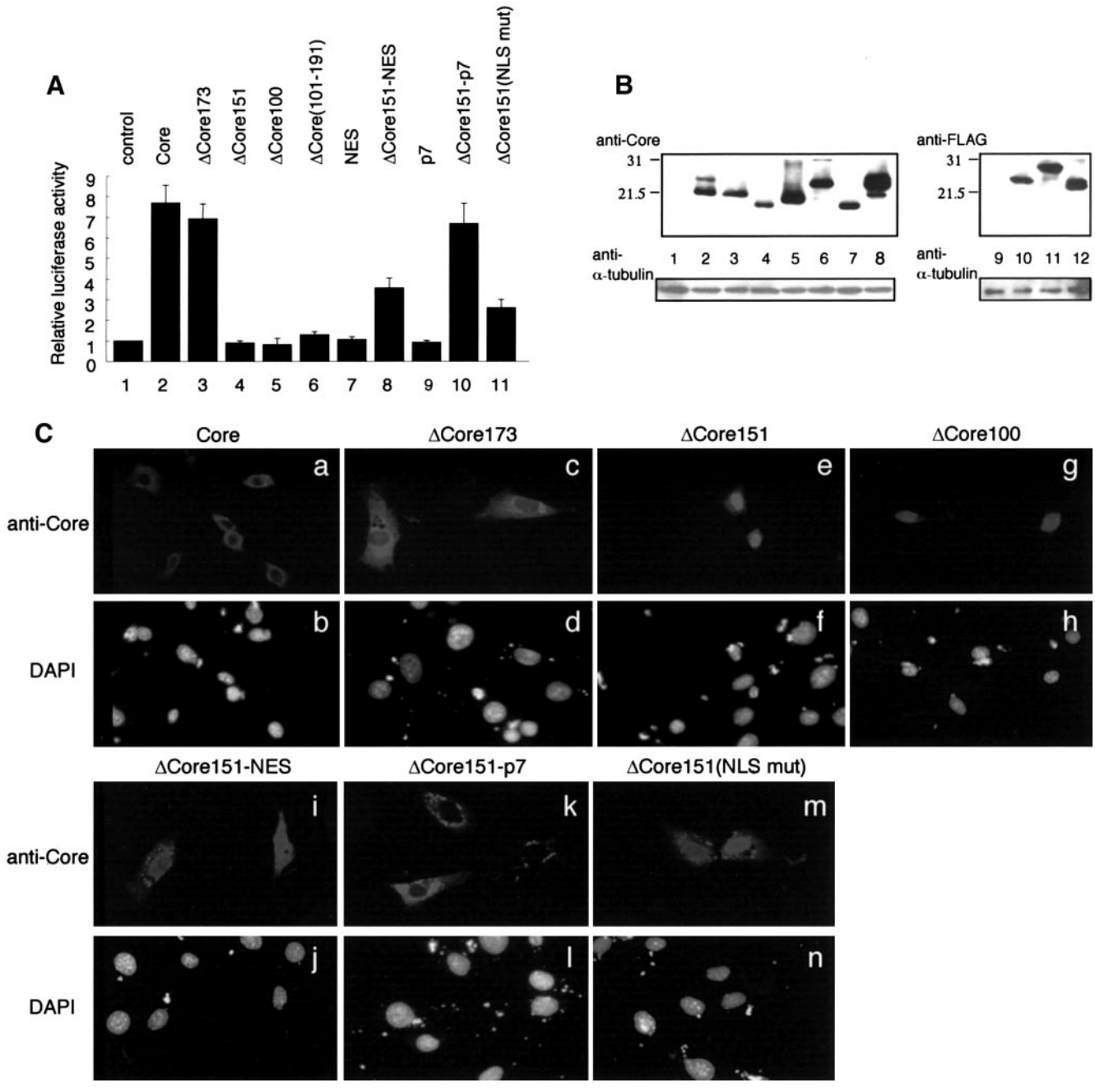

FIG. 1. Effect of altered cytoplasmic localization of the C-terminally truncated core on NF-κB activation capacity. (A) NF-κB activation capacity of the core mutants. NIH3T3 cells (1×10^4) were transfected with 0.05 μg of pRL-TK, 0.05 μg of pNF-κB-Luc, and 0.5 μg of various expression plasmids (pKS+/CMV in lane 1, pCMV-Core in lane 2, pCMV-ΔCore173 in lane 3, pCMV-ΔCore151 in lane 4, pCMV-ΔCore100 in lane 5, pCMV-ΔCore(101–191) in lane 6, pCMV-NES in lane 7, pCMV-ΔCore151-NES in lane 8, pCMV-p7 in lane 9, pCMV-ΔCore151-p7 in lane 10, and pCMV-ΔCore151 (NLS mut) in lane 11) with 1.2 μl of FuGENE 6. After 40 h, a whole cell lysate was prepared and assayed for firefly and *Renilla* luciferase activity. Luciferase activities for the index of NF-κB activity were calculated with firefly luciferase activity divided by *Renilla* luciferase activity. The data represent the mean of this relative luciferase activities from three independent experiments. The values of standard deviation are shown. (B) Expression levels of the core mutants produced in NIH3T3 cells. NIH3T3 cells (3×10^4) were transfected with 2.0 μg of each expression plasmid by using 3.0 μl of FuGENE 6. At 40 h posttransfection, the level of production of the core mutants was analyzed by SDS–polyacrylamide gel electrophoresis followed by immunoblotting analysis. The upper left and right panels show the proteins detected by anti-core (515S) and anti-FLAG, respectively. Molecular mass markers (in kilodaltons) are shown on the left. The lower panels indicate the production level detected by using anti-α-tubulin as an internal control. Plasmids which were transfected into the cells were as follows: Lane 1 and 9, pKS+/CMV; lane 2, pCMV-Core; lane 3, pCMV-ΔCore173; lane 4, pCMV-ΔCore151; lane 5, pCMV-ΔCore151-NES; lane 6, pCMV-ΔCore151-p7; lane 7, pCMV-ΔCore151(NLS-mut); lanes 8 and 12, pCMV-FLAG-Core; lane 10, pCMV-DHFR-FLAG-NES; lane 11, pCMV-DHFR-FLAG-p7. (C) Subcellular localization of the core mutants. NIH3T3 cells transfected with each expression plasmid pCMV-Core in a and b, pCMV-ΔCore173 in c and d, pCMV-ΔCore151 in e and f, pCMV-ΔCore100 in g and h, pCMV-ΔCore151-NES in i and j, pCMV-ΔCore151-p7 in k and l, and pCMV-ΔCore151 (NLS-mut) in m and n are shown. At 40 h posttransfection, the cells were fixed, permeabilized with 0.05% Triton X-100, and stained with anti-core antibody, and the immunocomplexes were detected with Alexa 568 goat anti-mouse IgG (H + L) conjugates (a, c, e, g, i, k, and m). Staining of the nucleus by DAPI in the same cells is shown in b, d, f, h, j, l, and n.

expected that a nuclear protein is exported to the cytoplasm by fusion with the NES sequence. The second modification was introduced by HCV p7 protein used as the C-terminal fusion partner. HCV p7 is considered to function as a signal sequence for NS2 protein during the proteolytic processing of HCV precursor polyprotein (Lin *et al.,* 1994; Mizushima *et al.,* 1994a,b). As secondary cleavage in p7 polypeptide was not observed (Lin *et al.,* 1994; Mizushima *et al.,* 1994b), the fused p7 was expected to function as an anchor for localization of the fusion protein on the surface of ER, similar to the wild-type core protein. For the third modification, we introduced a single amino acid mutation in the putative NLS of the core in order to render it inactive. Loss of NLS function was expected to result in the alteration of localization of the truncated core C151 to the cytoplasm. A previous report suggested that the region aa 38–43 of the core is the most active among the three or four putative NLSs (Chang *et al.,* 1994; Suzuki *et al.,* 1995). Consequently, to inactivate NLS, we substituted arginine for threonine at position 40 of the C151 protein (C151 (NLS mut)).

After the confirmation of protein production by immunoblotting analysis (Fig. 1B, lanes 5–7), we observed the subcellular localization of each construct by indirect immunofluorescence analysis. NES-fused C151 (C151-NES) and C151 (NLS mut) were distributed in both the nucleus and the cytoplasm in contrast to the nuclear localization of C151 (Fig. 1Ce, 1Ci, and 1Cm). In the case of C151-p7, alteration of the location was more obvious. As shown in Fig. 1Ck, the nuclear localization of C151 was changed to the perinuclear region in the cytoplasm by fusion with the signal sequence. Next, we examined NF-κB activity in the cells producing these proteins. As negative controls, NF-κB activity was evaluated in the cells transfected with the plasmid producing FLAG-tagged NES and FLAG-tagged p7 without the core polypeptide and with pCMV-DHFR-FLAG-NES and pCMV-DHFR-FLAG-p7 producing FLAG-tagged NES and FLAG-tagged p7 fused with mouse dihydrofolate reductase (DHFR), respectively. DHFR is a cytoplasmic cellular protein similar in molecular size to the core. Although the level of reporter activity in the cells producing negative control constructs was quite similar to that in the cells producing C151, the cells producing C151-NES, C151-p7, and C151 (NLS mut) showed 4.1-, 9.6-, and 3.3-fold, respectively, higher luciferase activities than the cells producing C151 (Fig. 1A, lanes 4 and 7–11). These data indicated that C151 has the potential to activate NF-κB when it is located in the cytoplasm. Similar results were obtained using HepG2 cells (data not shown). From the above results, the following conclusions have been reached: (1) cytoplasmic localization of the core is important for its NF-κB activation capacity, (2) the N-terminal region of 151 aa rather than the C-terminus of 40 aa of the core is required for the NF-κB activation, (3) the C-terminal region of the core functions to retain this protein in the cytoplasm.

The core is exposed on the cytosolic side of the endoplasmic reticulum membranes

As shown in Fig. 1A, the NF-κB activity of the cells producing C151-NES and C151 (NLS mut) was relatively weaker than that producing C191, whereas C151-p7 showed the same activation capacity as C191. As the production level of each protein was almost the same, the difference found in the effect of C151-p7 from those of C151-NES or C151 (NLS mut) seemed to be due to the different subcellular localization of this product. While C151-NES and C151 (NLS mut) were detected in both the nucleus and the cytoplasm, we observed that C151-p7 is located mainly around the perinuclear region, the same as C191 (Fig. 1Ci, 1Ck, and 1Cm). Therefore, we presume that NF-κB activation by the core is closely related to its localization around the perinuclear region. Since both C191 and C151-p7 have signal sequences in their C-termini, these proteins are likely to be located on endoplasmic reticulum (ER) membranes. Moreover, the core was suggested to be located on the outside of microsomal membranes by *in vitro* processing analysis for HCV structural proteins (Hijikata *et al.,* 1991). In order to investigate this point precisely, we analyzed the subcellular localization of C191 and C151-p7 by an indirect immunofluorescence technique. In this experiment, we used Grp78, which is also termed Bip and known to be located mainly in the lumen of ER (Haas, 1994), as a marker for ER. At first, NIH3T3 cells transfected with pCMV-Core or pCMV-ΔCore151-p7 were permeabilized with Triton X-100 for detection of the exogenous core derivatives and endogenous Grp78 by anti-core antibody and anti-Grp78 antibody, respectively. As shown in Fig. 2, immunofluorescence patterns for C191 and C151-p7 were similar to that for Grp78 (Figs. 2a, 2c, 2g, and 2i), suggesting that these proteins are located around the ER. Next, to investigate on which side of the ER membranes, the lumen or cytoplasmic side, C191 and C151-p7 are located, we performed a modified indirect immunofluorescence analysis in which digitonin was used for permeabilization of the cells instead of Triton X-100. Digitonin is a reagent that permeabilizes the plasma membranes effectively but not the intracellular membranes (Liu *et al.,* 1999; Pollenz *et al.,* 1992). Under these conditions, C191 and C151-p7 were detected around the perinuclear region (Figs. 2c, 2f, 2i, and 2l), just as in the cells treated with Triton X-100. However, we did not detect Grp78 in digitonin-permeabilized cells, the ER membranes of which should be largely intact to protect this protein from the antibody (Figs. 2a, 2d, 2g, and 2j). These results suggested that C191 and C151-p7 are exposed to the cytoplasm on the surface of the ER membranes.

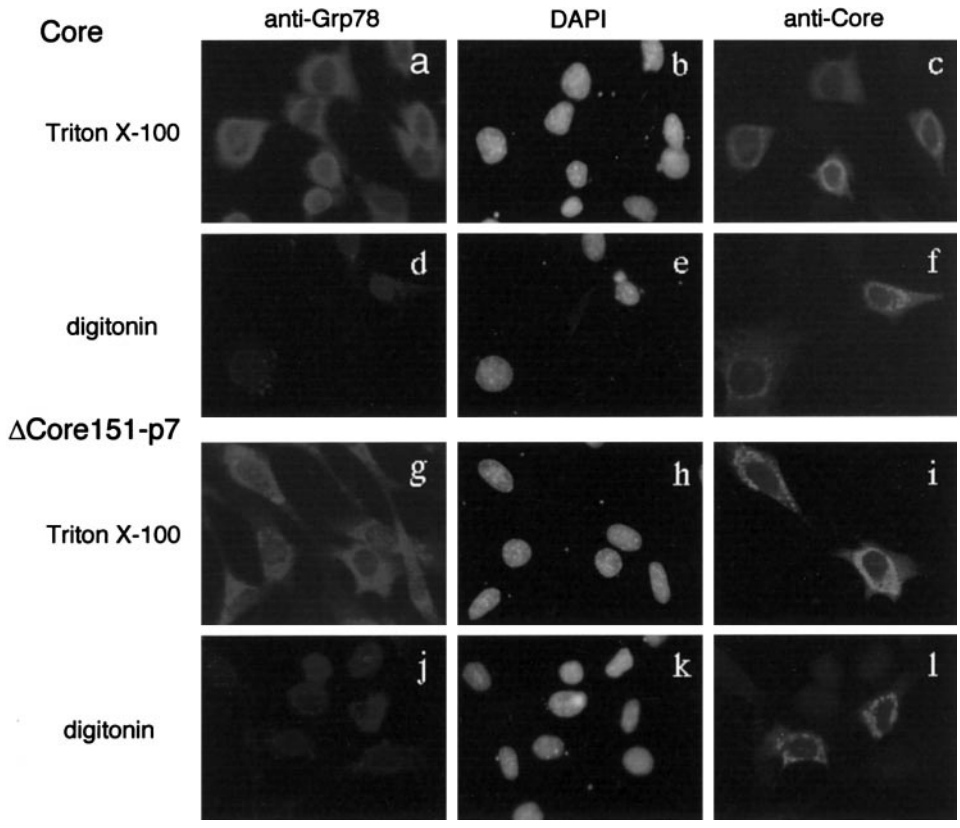

FIG. 2. Detection of Grp78 and the core after permeabilization with Triton X-100 or digitonin. NIH3T3 cells transfected with each expression plasmid pCMV-Core in a to f and pCMV-ΔCore151-p7 in g to l are shown. At 40 h posttransfection, the cells were fixed, permeabilized with 0.05% Triton X-100 (a, b, c, g, h, and i) or 40 μg/ml digitonin (d, e, f, j, k, and l), and stained with anti-core antibody and anti-Grp78 antibody, and the immunocomplexes were detected with Alexa 568 goat anti-rabbit IgG (H + L) conjugates (a, d, g, and j) and Alexa 488 goat anti-mouse IgG (H + L) conjugates (c, f, i, and l). Staining of the nucleus by DAPI in the same cells is shown in b, e, h, and k.

NF-κB activation by the core is closely related with ER localization of this viral protein

The above results suggested that localization of the core to ER membranes is important for NF-κB activation. To examine this possibility, we developed an experimental system in which subcellular localization of the core derivative can be conditionally changed from the cytoplasm to the surface of rough ER membranes. In this system, we used the expression plasmid for a fusion protein composed of the C-terminally deleted core which has a mutation in its NLS, HA epitope tag, and the N-terminal 212 aa of HCV NS3 (ΔCore-NS3.1) (Fig. 3). As the core part of this fusion construct, we used the region from aa 1 to 100 of the core. This region of the core entirely lost the C-terminal hydrophobic structures of this protein to reduce the affinity of this protein to the membranes. This region was confirmed to activate NF-κB when it was fused with FLAG-tagged p7. The N-terminal region of NS3 in ΔCore-NS3.1 was suggested to function for anchorage on the rough ER membrane through the interaction with HCV NS4A which was already reported to be localized on the membranes (Failla *et al.,* 1995; Hijikata *et al.,* 1993; Satoh *et al.,* 1995; Tanji *et al.,* 1995). Therefore, in the presence of NS4A, ΔCore-NS3.1 was expected to be located on the ER membranes through the association with this membrane protein. As a negative control, we prepared the fusion protein, ΔCore-NS3.2, which was identical to the ΔCore-NS3.1 except for the deletion of the NS4A binding region of ΔCore-NS3.1 corresponding to the N-terminal 22 aa of NS3 (Failla *et al.,* 1995; Satoh *et al.,* 1995) (Fig. 3). At first, we observed subcellular localization of these proteins in the presence or absence of NS4A (Fig. 4A). Both ΔCore-NS3.1 and ΔCore-NS3.2 were found to be distributed in the nucleus and cytoplasm in the absence of NS4A (Figs. 4Aa and 4Af), as observed in the case of C151 (NLS mut) (Fig. 1Cm). However, when ΔCore-NS3.1 was coproduced with NS4A, this fusion protein was found to accumulate around the perinuclear region and colocalize with NS4A in the cells (Figs. 4Ac and 4Ae). On the other hand, ΔCore-NS3.2 lacking the NS4A binding region was distributed in both the nucleus and the cytoplasm even in the presence of NS4A (Fig. 4Ah). When NS4A was produced in the cells, subcellular localization of NS4A was always observed to be around the perinuclear cytoplasmic region as reported previously (data not shown) (Selby *et al.,* 1993; Tanji *et al.,* 1995). These results indicated that ΔCore-NS3.1 was trapped around the rough

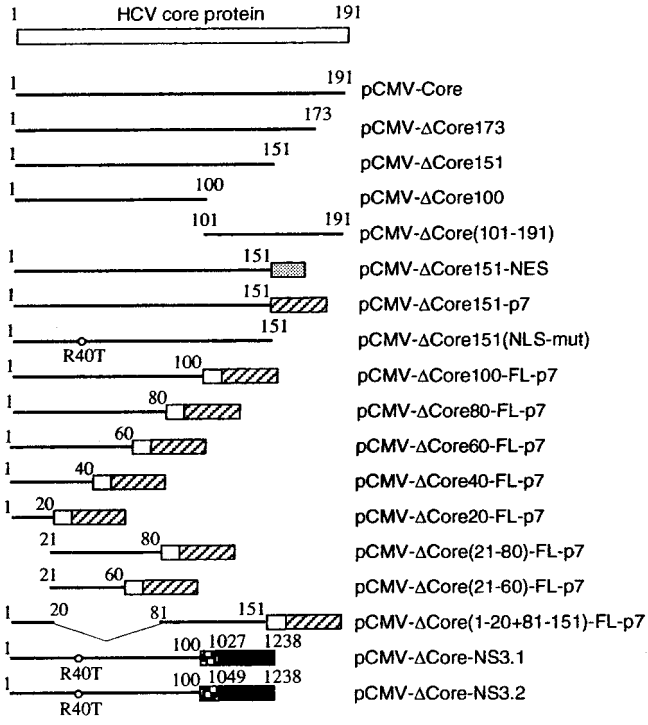

FIG. 3. Schematic representations of the HCV core protein derivatives encoded in the expression plasmid. The primarily processed form of the core is shown at the top. The core regions in deletion constructs are shown by bars. The numbers above the bars indicate the amino acid number of the viral precursor polyprotein. The shaded, hatched, open, checked, and closed boxes indicate the NES of HTLV-1 Rex, HCV p7, FLAG tag, HA tag, and HCV NS3, respectively.

ER membranes through the interaction with membrane-located NS4A. Under these conditions, we investigated the NF-κB activity in each cell. Compared with the negative control, no significant augmentation of NF-κB activation was found in the cells transfected with pCMV-ΔCore-NS3.1, pCMV-ΔCore-NS3.2, or pSPC-NS4A, indicating that these proteins do not have marked NF-κB activation capacities by themselves (Fig. 4B, lanes 2, 4, and 6). The NF-κB activity in the cells producing ΔCore-NS3.2 with NS4A was also the same as that in negative control cells (Fig. 4B, lanes 1 and 5). In contrast, about a 2.9-fold augmentation of NF-κB activity was found in the cells producing both ΔCore-NS3.1 and NS4A, compared with the cells producing ΔCore-NS3.1 without NS4A (Fig. 4B, lanes 2 and 3). We observed that the production level of each exogenous protein was almost identical in each transfectant (Fig. 4C). We also confirmed that both ΔCore-NS3.1 and ΔCore-NS3.2 proteins have the capacity to activate NF-κB when produced as forms fused with p7 to be located around the perinuclear cytoplasmic region (data not shown). From the above results, it was suggested that activation of NF-κB by the core is closely related to its localization to the rough ER membranes.

The region from aa 21 to 80 of the core is essential for efficient NF-κB activation

To identify the structure of the core which is involved in NF-κB activation, we examined the activation capacity of a variety of truncated cores fused with FLAG-tagged p7. As shown in Fig. 5, significant augmentation of NF-κB activity was found in the cells transfected with pCMV-ΔCore100-p7, pCMV-ΔCore80-p7, and pCMV-ΔCore60-p7, compared to the negative controls, FLAG-p7 and DHFR-FLAG-p7, whereas pCMV-ΔCore40-p7 and pCMV-ΔCore20-p7 showed reduced NF-κB activation potentials (Fig. 5, lanes 3–8 and 12). The reduced potentials seemed to be marginal, because ΔCore40-p7 and ΔCore20-p7 did not activate NF-κB in HepG2 cells at all, despite reproduced observations of the augmentation of NF-κB activity by ΔCore100-p7, ΔCore80-p7, and ΔCore60-p7 in the cells (data not shown). To narrow down the core region required for the NF-κB activation, another deletion mutant was examined. We found intermediate activation of NF-κB in the cells producing aa 21–60 of the core (Fig. 5, lane 10). We confirmed that the production levels and perinuclear localizations of these chimeric proteins were similar to each other in NIH3T3 and HepG2 cells by immunoblotting and indirect immunofluorescence analyses (data not shown). These data suggested that the region from aa 21 to 60 of the core is essential for the efficient activation of NF-κB. In addition, when the region from aa 61 to 80 was lost, NF-κB activity was reduced (Fig. 5, lanes 5, 6, 9, and 10). Therefore, it was supposed that the region aa 61–80 of the core plays a supportive role in NF-κB activation. To test the above conclusion, we examined the potential of the p7-fused C151 lacking aa 21–80 of the core and found no NF-κB activation in the cells producing that deletion construct (Fig. 5, lane 11).

DISCUSSION

In this study, we showed that the cytoplasmic localization of the core is essential for the activation of NF-κB by this viral protein. We also obtained data suggesting that the core is localized to the ER membranes exposing some parts to the cytoplasm. Moreover, we revealed that the localization of the core to the membranes of rough ER is important for the activation of NF-κB. This suggested that activation by the core occurred in the cytoplasm rather than in the nucleus, although nuclear localization of the core was reported (Yasui *et al.,* 1998). It is well-established that one of the major regulatory mechanisms for NF-κB activity is present in the cytoplasm as follows. Under dormant conditions, NF-κB activity is negatively regulated by the formation of a complex with IκB in the cytoplasm. NF-κB activation signals induce phosphorylation and subsequent degradation of IκB through the kination cascade including IκB kinase (IKK) α, β, and γ, resulting in dissociation of NF-κB from IκB. Subse-

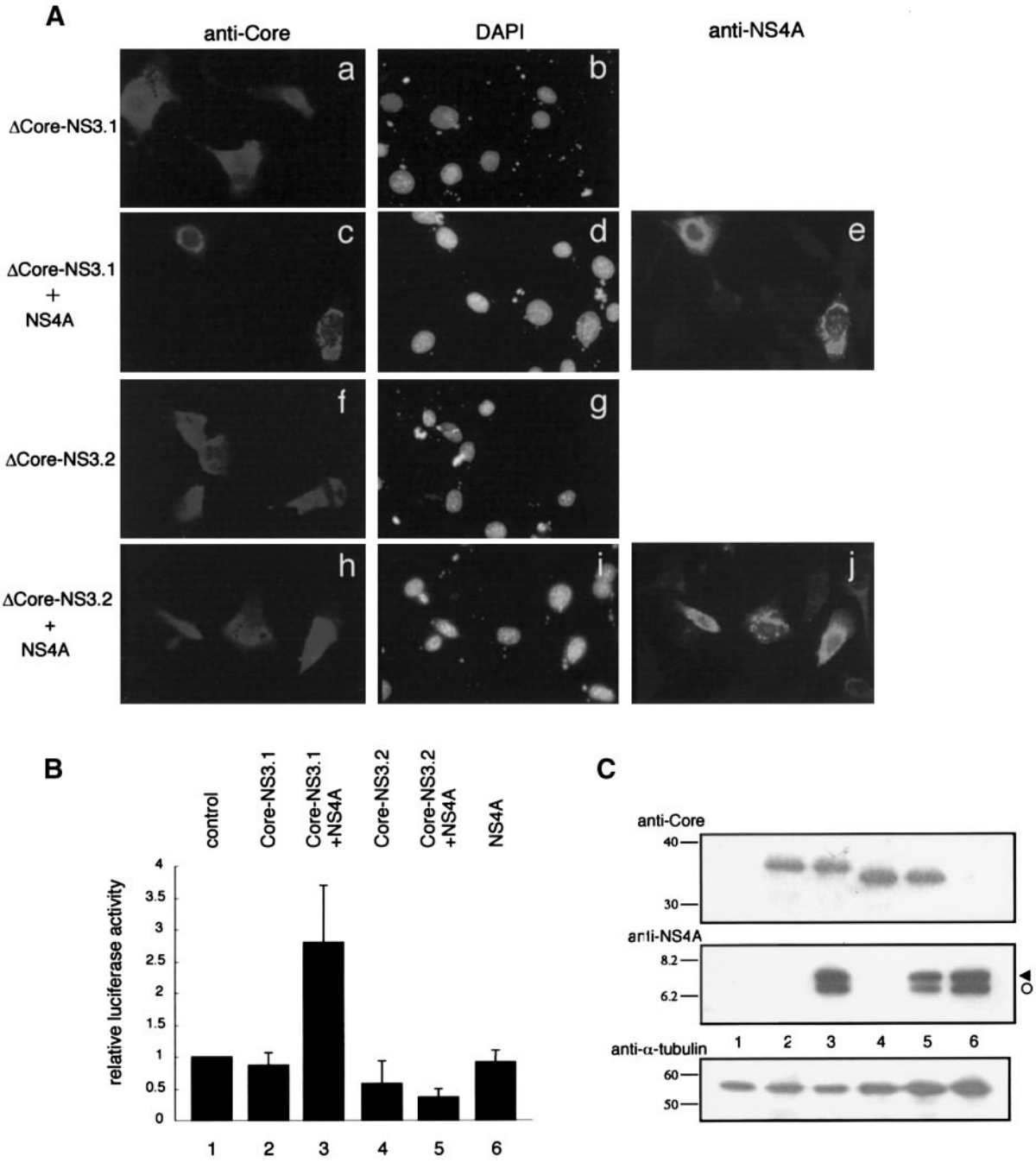

FIG. 4. Effect of conditional localization of the mutated core around the ER membrane on NF-κB activation capacity. (A) Subcellular localization of the core-NS3 chimera in the presence of NS4A. A total of 1.0 μg of pCMV-ΔCore-NS3.1 and 0.25 μg of pKS+/CMV (a and b), 1.0 μg of pCMV-ΔCore-NS3.1 and 0.25 μg of pSPC-NS4A (c, d, and e), 1.0 μg of pCMV-ΔCore-NS3.2 and 0.25 μg of pKS+/CMV (f and g), and 1.0 μg of pCMV-ΔCore-NS3.2 and 0.25 μg of pSPC-NS4A (h, i, and j), respectively, were transfected into NIH3T3 cells. The immunocomplexes reactive with anti-core and anti-NS4A antibody were detected with Alexa 568 (left) and Alexa 488 (right) goat anti-mouse IgG (H + L) conjugates, respectively. DAPI was also used for the nuclear staining (center). (B) NF-κB activation capacity when the core-NS3 chimera was produced with or without NS4A. A total of 0.4 μg of pCMV-ΔCore-NS3.1 or pCMV-ΔCore-NS3.2 was transfected into NIH3T3 cells with 0.1 μg of pSPC-NS4A or pKS+/CMV. The luciferase activities were measured as described in the legend to Fig. 1A. The results of the cells transfected with pKS+/CMV, pCMV-ΔCore-NS3.1, pCMV-ΔCore-NS3.1 and pSPC-NS4A, pCMV-ΔCore-NS3.2, pCMV-ΔCore-NS3.2 and pSPC-NS4A, and pSPC-NS4A were shown in lanes 1, 2, 3, 4, 5, and 6, respectively. (C) Production levels of the core-NS3 chimera and NS4A in NIH3T3 cells. NIH3T3 cells were transfected as indicated for (B). Immunoblotting analyses were performed as described for Fig. 1B for the detection of the expression levels of the core mutants (top), NS4A (middle), and α-tubulin (bottom) were used as the internal controls. Molecular mass markers (in kilodaltons) are shown on the left. The arrow indicates NS4A protein. The open circle represents a possible degradation product of NS4A.

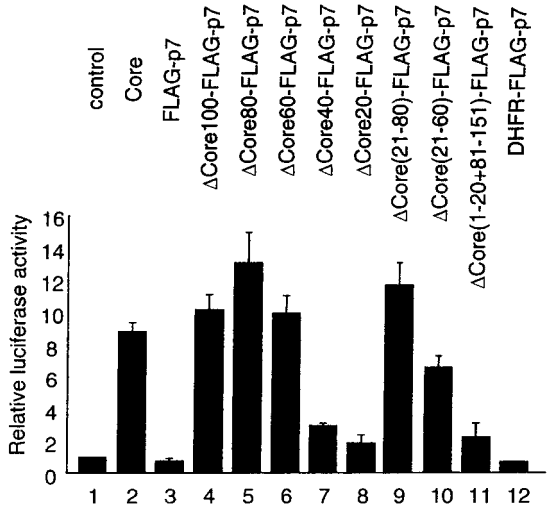

FIG. 5. The identification of the region of the core involved in NF-κB activation. NIH3T3 cells were transfected with 0.5 μg of each expression plasmid, pKS+/CMV (lane 1), pCMV-Core (lane 2), pCMV-FLAG-p7 (lane 3), pCMV-ΔCore100-FLAG-p7 (lane 4), pCMV-ΔCore80-FLAG-p7 (lane 5), pCMV-ΔCore60-FLAG-p7 (lane 6), pCMV-ΔCore40-FLAG-p7 (lane 7), pCMV-ΔCore20-FLAG-p7 (lane 8), pCMV-ΔCore(21–80)-FLAG-p7 (lane 9), pCMV-ΔCore(21–60)-FLAG-p7 (lane 10), pCMV-ΔCore(1–20 + 81–151)-FLAG-p7 (lane 11), and pCMV-DHFR-FLAG-p7 (lane 12). The luciferase activities were measured as indicated for Fig. 1A.

quently, the released NF-κB enters the nucleus and induces the expression of numerous genes (Karin, 1999; Pahl, 1999). Therefore, our results are likely to indicate that the core modulates a certain step of this cytoplasmic NF-κB signaling pathway.

Recently, it has been reported that some viral proteins such as adenovirus E3/19K protein and influenza virus hemagglutinin protein caused NF-κB activation by overaccumulation in the ER lumen (Pahl and Bauerle, 1995, 1997; Pahl *et al.,* 1996). This NF-κB activation, so-called ER overload, is considered to be triggered by the release of calcium cations from the ER and followed by production of reactive oxygen species (Pahl *et al.,* 1997). However, this typical ER overload did not seem to be involved in NF-κB activation by the core for the following reasons. First, NF-κB activation by ER overload is caused by sensing an overaccumulation of proteins in the ER lumen. However, the region of aa 21–80 of the core seemed to be present on the cytoplasmic side of the ER, not in the lumen, because the epitope of the anti-core antibody used in this study was located among aa 21–40 of the core, which was included in the above functional region of the core. Second, although it was reported that the ER stress causes not only the activation of NF-κB but also the reduction of translational activity in the cells, we found no remarkable reduction in translation and monitored the increased production level of the luciferase induced by the core production. Third, the core mutant, which did not show any affinity to the membranes, activated NF-κB when it was localized around the ER membranes through interaction with NS4A, which does not activate NF-κB.

Until now, there has been no report suggesting that any of the NF-κB activation factors such as the IKK complex are located around the ER membrane. It seems possible, however, that the localization of the core to the ER membranes creates an opportunity for interaction with a cellular factor leading to NF-κB activation. Recently, it was suggested from the results obtained by a yeast two-hybrid system that the core interacted with the intracellular domain of lymphotoxin-β receptor (LT-βR) and tumor necrosis factor receptor 1 (TNFR 1) (Chen *et al.,* 1997; Matsumoto *et al.,* 1997; Zhu *et al.,* 1998). Generally, integral membrane proteins such as ligand receptors of plasma membranes are translated by the ribosomes on the rough ER and their extracellular portions are translocated into the lumen of ER cotranslationally. These primary translation products are subsequently glycosylated at the portion in the lumen and transported to the plasma membrane through the Golgi apparatus and endosomal network. This means that the core may have an opportunity to interact with the cytoplasmic region of these newly synthesized plasma membrane proteins. This type of molecular interaction on the intracellular membrane has been recently reported. A cellular factor known as the silencer of death domain (SODD) was found to interact with the cytoplasmic domain of TNFR 1 and locate around the Golgi apparatus (Jiang *et al.,* 1999). It was suggested that SODD bound to TNFR 1 is translocated to the plasma membrane and suppresses the signal from TNFR 1 introduced by TNF, although the majority of SODD was found to be located around the Golgi apparatus. The core was also reported to associate with TNFR 1 (Zhu *et al.,* 1998), although the core has no significant amino acid sequence homology to the SODD. Matsumoto *et al.* showed that the region from aa 36 to 91 of the core binds to LT-βR (Matsumoto *et al.,* 1997). Chen *et al.* also reported that the N-terminal 40-aa region of the core is important for the interaction with LT-βR by *in vitro* binding analysis using a recombinant truncated core protein fused to GST (Chen *et al.,* 1997). The former LT-βR interaction region of the core almost overlapped with aa 21–80 of the core which was observed to be involved in the NF-κB activation in this paper. Therefore, it is possible that the core activates NF-κB through the interaction with these receptor molecules. Although there have been no reports indicating that the core is located on the inner surface of the plasma membranes, a part of the core bound with those receptor molecules may be transported to the plasma membranes and enhance the signal of NF-κB activation, in contrast to SODD. Then, how is the core supposed to function for NF-κB activation when it binds to TNFR 1? One possibility is that the core may function like TRADD and enhance NF-κB activity. TRADD is known as an adaptor molecule for TNFR 1 and TRAF 2 and has been

suggested to mediate signal transduction for NF-κB activation by TNFα stimulation (Karin, 1999). Another possibility may be that the core competes for binding to TNFR 1 with SODD which suppresses NF-κB activation. Further studies are necessary to determine whether the interaction between the core and TNFR 1 or LT-βR is correlated with the activation of NF-κB by the core and whether there is other candidate(s) that associates with the core for the activation after the ER membrane localization of the core.

To see whether the activation region of the core shares characteristic features with other proteins which have been reported to be involved in activation of NF-κB, a homology analysis of the amino acid sequence was performed. The results showed that the region of the Tax protein corresponding to aa 61–74 of HTLV-1 (GRVIGSALQFLIPR) has 71% similarity with the core in the region of aa 28–39 (GQIVGGVYLLPR). Tax has been reported to be associated with plural targets, NF-κB subunits, IκB, IKKγ, MEK kinase 1, and NF-κB inducing kinase (NIK) among the molecules composing the NF-κB signaling pathway (Sun and Ballard, 1999). This may imply that the core shares some targets for the NF-κB activation with Tax, although the subcellular localization of Tax seems to be different from that of the core. However, our preliminary data showed no interaction between the core and NIK or IkBα *in vitro* (data not shown). Further analyses to see whether this region of the core is associated with cellular factors including these molecules are now underway.

We previously suggested that NF-κB activation is part of the anti-apoptotic effect of the core (Marusawa *et al.,* 1999). Moreover, as NF-κB is suggested to play an important role in the transformation of cells or known to regulate a variety of inflammatory cytokines (Pahl, 1999; Yamaoka *et al.,* 1996), NF-κB activation by the core is likely to be involved in the hepatocarcinogenesis or the development of HCV diseases. In order to determine the cause of these diseases, further analysis on the mechanism of NF-κB activation by the core is required.

MATERIALS AND METHODS

Plasmid constructs

The expression plasmids used in this study are summarized and schematically represented in Fig. 3. These plasmids were constructed by insertion of the DNA fragments amplified by polymerase chain reaction (PCR) using Pfu polymerase (Promega) or synthesized oligonucleotides into the pKS+/CMV vector (Hijikata *et al.,* 1993). pCMV-Core, pCMV-ΔCore173, and pCMV-ΔCore151 were constructed as described previously (Marusawa *et al.,* 1999). pCMV-ΔCore100 was constructed with the fragment amplified by PCR using oligonucleotides core-s (Marusawa *et al.,* 1999) and 5′-ACAGAATTCTAGGGTGACAGGAGCCATC-3′ as primers and pCMV-Core as the template. pCMV-ΔCore (101–191) was generated by inserting the PCR product obtained using the oligonucleotides 5′-GTTGGATCCATGCGTGGCTCCCGGCCTAATTG-3′ and 5′-CTCGAATTCTCAAGCGGAAGCTGGGATGGTCA-3′ as primers into pKS+/CMV in the same manner. pCMV-ΔCore151-NES and pCMV-ΔCore151-p7 were produced by ligating PCR products A and B, respectively, into *Eco*RI–*Hin*dIII-digested pCMV-ΔCore151. PCR product A was prepared by using pRSV-Tax (Hirowatari *et al.,* 1995) as the template and the oligonucleotides 5′-ACAGAATTCGCGTTATCGGCTCA-3′ and 5′-TTCAAGCTTTCAGGAGTCGAGGGATA-3′ as primers. PCR product B was obtained by using pCMV-3010 (Marusawa *et al.,* 1999) as the template and the oligonucleotides 5′-ACAGAATTCGGCCTTCTCTCCTTCCTCGTGTT-3′ and 5′-TTCAAGCTTTCAGGCATAAGCTCGTGGTGGTAA-3′ as primers (the latter oligonucleotide was named p7-AS). pCMV-ΔCore151 (NLS mut) was produced by oligonucleotide-directed mutagenesis using 5′-TTGCCGCGCACGGGCCCCAGGT-3′ and 5′-ACCTGGGGCCCGTGCGCGGCAA-3′ as primers (Mizushima *et al.,* 1994a). The fragment amplified by PCR with oligonucleotide 5′-ACAGAATTCCACAGATCTGGCCTTCTCTCCTTCCTCGTGTT-3′ and p7-AS as primers and pCMV-3010 as a template was ligated into the *Eco*RI–*Hin*dIII sites of the pKS+/CMV vector and the resultant plasmid was named pCMV-p7. Subsequently, pCMV-FLAG-p7 was generated by ligating the fragment prepared by annealing oligonucleotides 5′-AATTCATGGATTACAAGGATGACGACGATAAGA-3′ and 5′-GATCTCTTATCGTCGTCATCCTTGTAATCCATG-3′ into the *Eco*RI–*Bgl*II sites of pCMV-p7. pCMV-ΔCore100-FLAG-p7, pCMV-ΔCore80-FLAG-p7, pCMV-ΔCore60-FLAG-p7, pCMV-ΔCore40-FLAG-p7, pCMV-ΔCore20-FLAG-p7, pCMV-ΔCore(21–80)-FLAG-p7, pCMV-ΔCore(21–60)-FLAG-p7, and pCMV-ΔCore(1–20 + 81–151)-FLAG-p7 were prepared by replacing the *Bam*HI–*Eco*RI fragment of pCMV-FLAG-p7 with the *Bam*HI–*Eco*RI digests of PCR products C, D, E, F, G, H, I, and J, respectively. The PCR products C, D, E, F, G, H, and I were amplified by using pCMV-Core as a template and oligonucleotides core-s and 5′-ACAGAATTCGGGTGACAGGAGCCATC-3′, 5′-ATAGAATTCCCCGGGCTGAGCCCAGGTCC-3′ (core80-AS), 5′-TGTGATTCTCCACGAGGTTGCGA-3′ (core60-AS), 5′-TGTGAATTCCCTGCGCGGCAACAGGTAAA-3′, and 5′-TGTGAATTCCTGTGGGCGGCGGTTGGTGTTA-3′, for C, D, E, F, and G, respectively, oligonucleotides 5′-TCTGGATCCATGGACGTTAAGTTCCC-3′ (Core21-S) and core80-AS for H, and core21-S and core60-AS for I as primers. The PCR product J was obtained through a two-step PCR reaction. The first PCR products were obtained using core-s and oligonucleotides 5′-GAGGGGCCAAGGGTACTGTGGGCGGCGGTT-3′ for the PCR product named J1 and 5′-ACTGAATTCCAGGGCCCTGGCAACGCCTC-3′ (named core151-AS) and 5′-AACCGCCGCCCACAGTACCCTTGGCCCCTC-3′

for the PCR product J2 as primers and pCMV-Core as a template. The second PCR was performed with J1 and J2 as templates and core-s and core151-AS as primers to obtain the J PCR product. pCMV-ΔCore-NS3.1 and pCMV-ΔCore-NS3.2 were produced by inserting three fragments generated as follows into pCMV-FLAG-p7. Initially, the fragment synthesized by annealing oligonucleotides 5′-AATTCTACCCATACGATGTTCCAGATTACGCTA-3′ and 5′-GATCTAGCGTAATCTGGAACATCGTATGGGTAG-3′ was inserted into the *Eco*RI–*Bgl*II sites of pCMV-FLAG-p7 and the resultant plasmid was named pCMV-HA-p7. Next, the PCR product obtained with oligonucleotides 5′-TTGTGGTTGAGATCTGCGCCTATCACGGCCTATTC-3′ and 5′-TTGTGGTTGAAGCTTTCAGGTGCTCTTGCCGCTGCCAG-3′ (NS3-AS) for pCMV-ΔCore-NS3.1 and oligonucleotides 5′-ACTGGTTGAGATCTGGTCGGGACAAGAA C-3′ and NS3-AS for pCMV-ΔCore-NS3.2 as primers was replaced with the *Bgl*II–*Hin*dII digests of pCMV-HA-p7 (these resultant plasmids were named pCMV-NS3.1 and pCMV-NS3.2, respectively). The third insert was amplified using core-s and 5′-ACAGAATTCGGGTGACAGGAGCCATC-3′ as primers and pCMV-ΔCore151 (NLS mut) as the template, followed by insertion into pCMV-NS3.1 and pCMV-NS3.2 to produce pCMV-ΔCore-NS3.1 and pCMV-ΔCore-NS3.2, respectively.

Cell culture

NIH3T3 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Nissui) with 10% fetal bovine serum and L-glutamine at 37°C.

Transfection of cells

The DNA transfection experiment was performed using FuGENE 6 transfection reagent (Boehringer Mannheim) as described previously (Marusawa *et al.,* 1999).

Reporter plasmid assay

Luciferase activity was measured by a luminometer with a dual-luciferase reporter assay system kit (Promega) as recommended by the manufacturer. The reporter pNF-κB-Luc (Stratagene) vector containing the NF-κB binding elements upstream of the minimum promoter region driving the firefly luciferase reporter gene was used. In this assay system, the *Renilla* luciferase activity from the pRL-TK (Promega) vector containing the herpes simplex virus thymidine kinase promoter upstream of the *Renilla* luciferase gene was also monitored to normalize firefly luciferase activity.

Immunoblotting analysis

The preparation of cell lysate, sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as described previously (Marusawa *et al.,* 1999). The antibodies used in this study were those against HCV core protein (515S; a generous gift from Dr. Kohara, Tokyo Metropolitan Institute of Medical Science), NS4A protein (anti-NS4A), and FLAG epitope (anti-FLAG M2, Sigma).

Immunofluorescence

The immunofluorescence analysis was performed as described previously (Marusawa *et al.,* 1999). The cells were permeabilized with 0.05% Triton X-100. The primary antibodies used in this experiment were 515S, anti-NS4A, anti-FLAG, or anti-Grp78 (StressGen Biotechnologies Corp.). The secondary antibodies were Alexa 568 goat anti-mouse IgG (H + L) and Alexa 488 goat anti-rabbit IgG (H + L) conjugates (Molecular Probes, Eugene, OR). The nucleus was stained using 4′, 6-diamidino-2-phenylindole (DAPI). In the experiment with digitonin, we treated the cells with buffer containing 20 mM HEPES–KOH (pH 7.3), 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, and 40 μg/ml digitonin on ice for 5 min instead of with 0.05% Triton X-100.

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